Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

One-step DNA-programmed growth of CpG conjugated silver nanoclusters: a potential platform for simultaneous enhanced immune response and cell imaging

Experimental Section

Materials and Instrumentation: Silver nitrate ($AgNO_3$, 99.9995%) and sodium borohydride ($NaBH_4$, 98%) were purchased from Alfa Aesar and used without further purification. Purified anti-mouse TNF-α, Biotin conjugated anti-mouse TNF-α cocktail, Anti-mouse IL-6, Biotin anti-mouse IL-6, were obtained from eBioscience. (O-phenylenediamine) OPD substrate was purchased from DingGuo. Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (USA). Dulbecco's modified Eagle's medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Invitrogen. The mouse leukemic monocyte macrophage cell line RAW264.7 cell line was obtained from Cell Bank of Chinese Academy of Sciences (Shanghai). All other reagents were of analytical reagent grade and used as received. Nanopure water (18.2 MΩ; Millpore Co., USA) was used in all experiments and to prepare all buffers. DNA oligonucleotides were synthesized by Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China). The sequences were as follows: CpG Oligodeoxynucleotides: 5'-TCC ATG ACG TTC CTG ACG TT-3'; DNA template for CpG-AgNCs: 5'-TCC ATG ACG TTC CTG ACG TTA AAA AAC CCC CCC CCC CC -3'; DNA template for AgNCs: 5'- CCC CCC CCC CCC -3'

Fluorescence measurements were carried out by using a JASCO FP-6500 spectrofluorometer (Jasco International Co., Japan). TEM images were recorded using a FEI TECNAI G2 20 high-resolution transmission electron microscope operating at 200 kV. Fluorescence images were captured using an Olympus BX-51 optical equiped with a CCD camera.

Preparation of CpG-AgNCs: The silver nanoclusters were synthesized by cooling the solution of DNA and $AgNO_3$ to 0 °C and then adding $NaBH_4$, followed by vigorous shaking for 2 min. Unless otherwise specified, final concentrations were 5 μM for the DNA template, 30 μM for $AgNO_3$, and 30 μM for $NaBH_4$. The solutions were kept in the dark prior to measurements. Experiments were carried out in 25 mM phosphate buffer at pH 7.0, unless otherwise noted. For pH-effect experiments, buffers with the desired pH value containing a total concentration of 25 mM phosphate were used.

Cell culture: The murine macrophage-like RAW264.7 cells were grown at 37 °C in an atmosphere of 5% (v/v) CO_2 in air., in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS, 1.5 g/L $NaHCO_3$, 100 units/ml penicillin, 100 mg/ml streptomycin, 4.5 g/L glucose and 4 mM glutamine. The media was changed every three days, and the cells were digested by trypsin and resuspended in fresh complete medium before plating.

Cytotoxicity assays: MTT assays were used to probe cellular viability. RAW264.7 cells were seeded at a density of 5000 cells/well (100 μL total volume/well) in 96-well assay plates. After 24 h incubation, the as-prepared CpG-AgNCs, at the

indicated concentrations, were added for further incubation of 48 h. To determine toxicity, 10 μL of MTT solution (BBI) was added to each well of the microtiter plate and the plate was incubated in the CO_2 incubator for an additional 4 h. Then the cells were lysed by the addition of 100 μL of DMSO. Absorbance values of formazan were determined with Bio-Rad model-680 microplate reader at 490 nm (corrected for background absorbance at 630 nm). In addition, the THP-1 cells were exposed to similar concentrations of CpG-AgNCs for 48 h. Culture was processed and subjected to MTT assay as discussed above. Untreated cells were used as a positive control (100% viable) in the study. Three replicates were done for each treatment group.

LDH Assay: The cell membrane integrity can be determined by lactate dehydrogenase (LDH) leakage assay. The activity of LDH in the medium was determined using a commercial LDH kit (Beyotime institute of biotechnology, China) according to the manufacturer's protocol. RAW264.7 cells and THP-1 cells were seeded at a density of 20,000 cells/well in 96-well plates and allowed to settle overnight. Next, cells were incubated with CpG-AgNCs at 0, 2.5, 10, 25, 100, 250 and 500 nM. In this experiment, cells lysed by 1% Triton-X-100 were used as the positive control, while the untreated cells were assayed as a negative control. After 48 h, cells were precipitated by centrifugation, and the culture medium was collected and treated with LDH Reaction Mix solution for 20 min at 25 °C. Then, the absorbance was measured in a microplate reader at 490 nm, with 620 nm set as the reference wavelength. The cytotoxicity as a percentage of dead cells was calculated using the

following equation: LDH leakage (%) = (sample − negative control) / (positive control − negative control) × 100.

Cell imaging: RAW264.7 cells were seeded onto 12-mm sterile coverslips in a 24-well plate. After 24 h, cells were incubated with ROX-CpG ODNs or CpG-AgNCs for two hours at 37 °C. To remove the unbound ODNs or AgNCs, the cells were washed three times with PBS. After that, the imagings were captured using an Olympus BX-51 optical equiped with a CCD camera.

Cytokine Assays: RAW264.7 cells were seeded on 24-well culture plates at a density of 5×10^5 cells/well. After 24 h incubation, cells were washed with 0.5 ml PBS before treatment with indicated conditions for 8 h (TNF-α) or 24 h (IL-6). The supernatants were collected and stored at -80 °C until use. The levels of TNF-α, and IL-6 in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using antibody pairs specific to these cytokines following protocols recommended by the manufacturer.

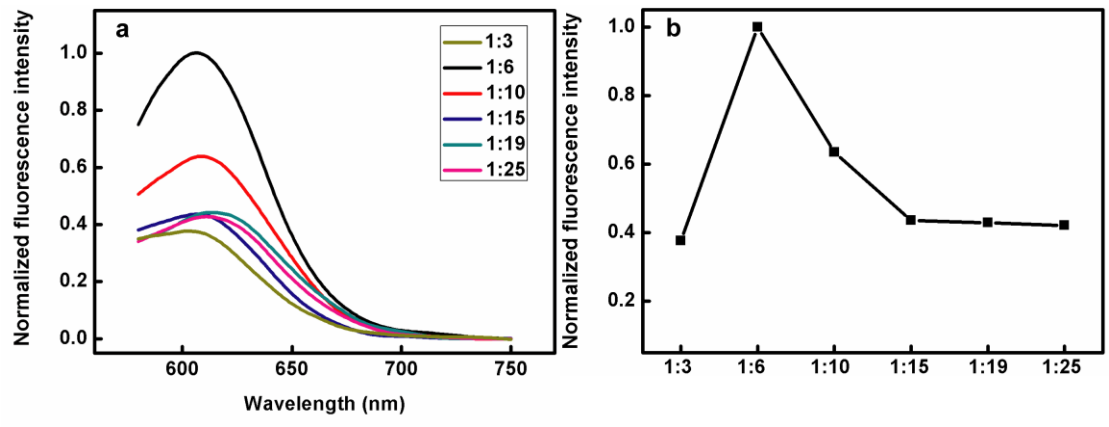

Fig. S1 Dependence of the fluorescence intensity with the stoichiometry of Ag^{+} : DNA base.

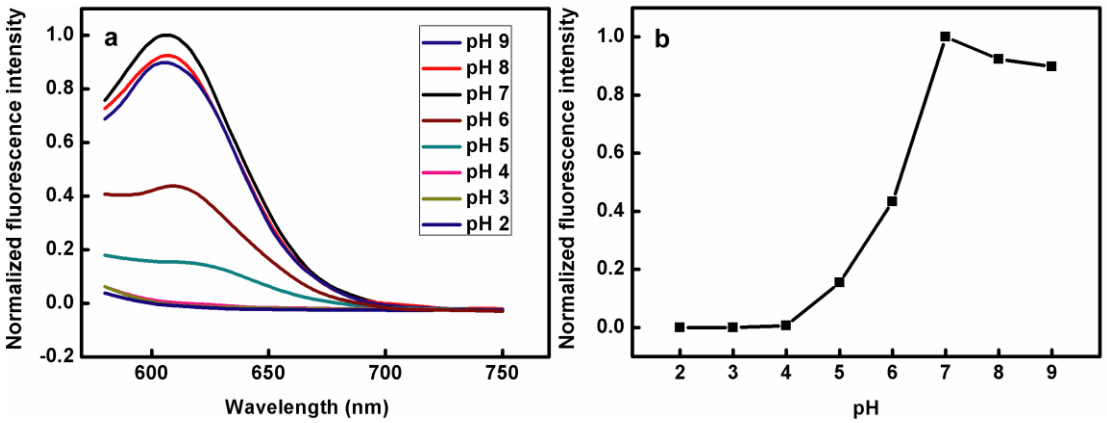

Fig. S2 Fluorescence emission spectra of DNA-Ag NCs at different pH.

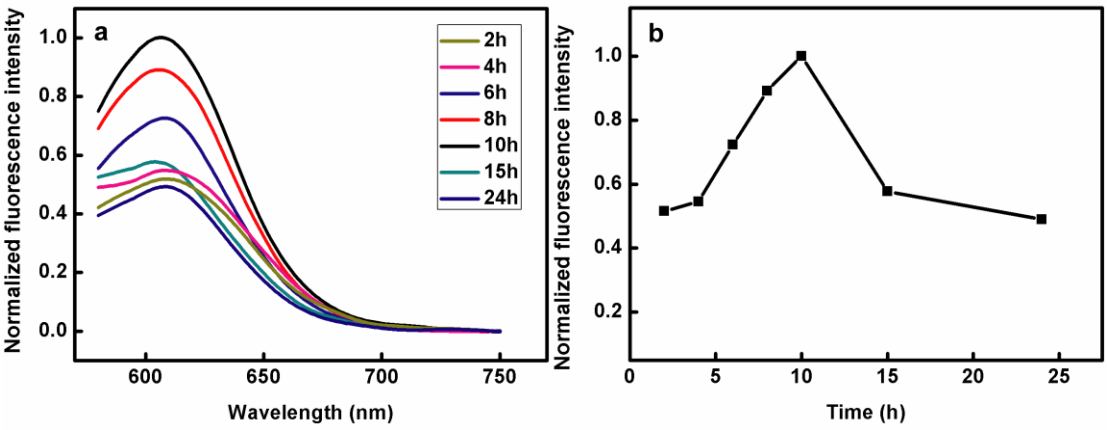

Fig. S3 Time evolution of the fluorescence intensities of the CpG-AgNCs.

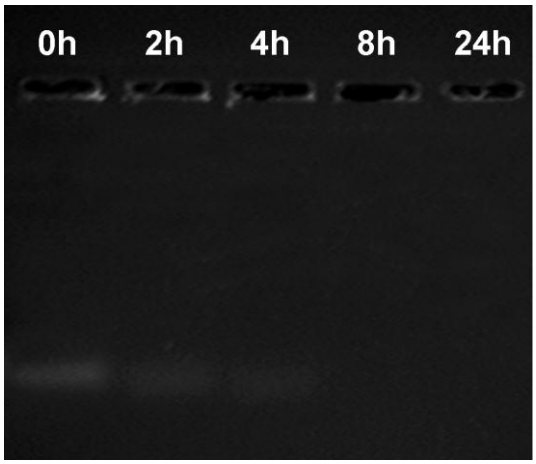

Fig. S4 Electrophoretic analysis of the stability of the CpG ODNs. The CpG ODNs were incubated in 50% non-heat-inactivated fetal bovine serum (FBS) at 37 ^{o}C for 2-24 h and then analyzed with gel electrophoresis.

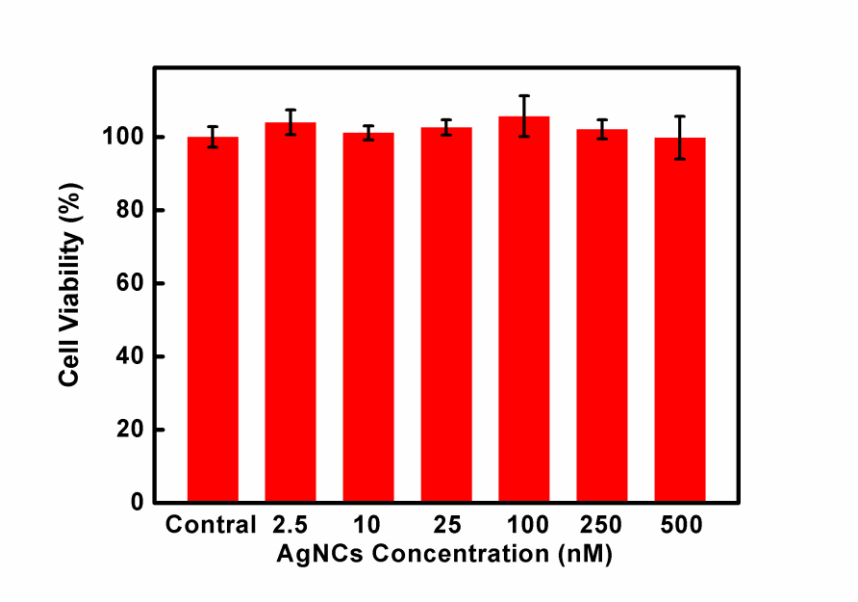

Fig. S5 Cytotoxicity of THP-1 cells evaluated by MTT assays after incubation with CpG-AgNCs with different concentrations for 48 h. The error bars represent variations among three independent measurements.

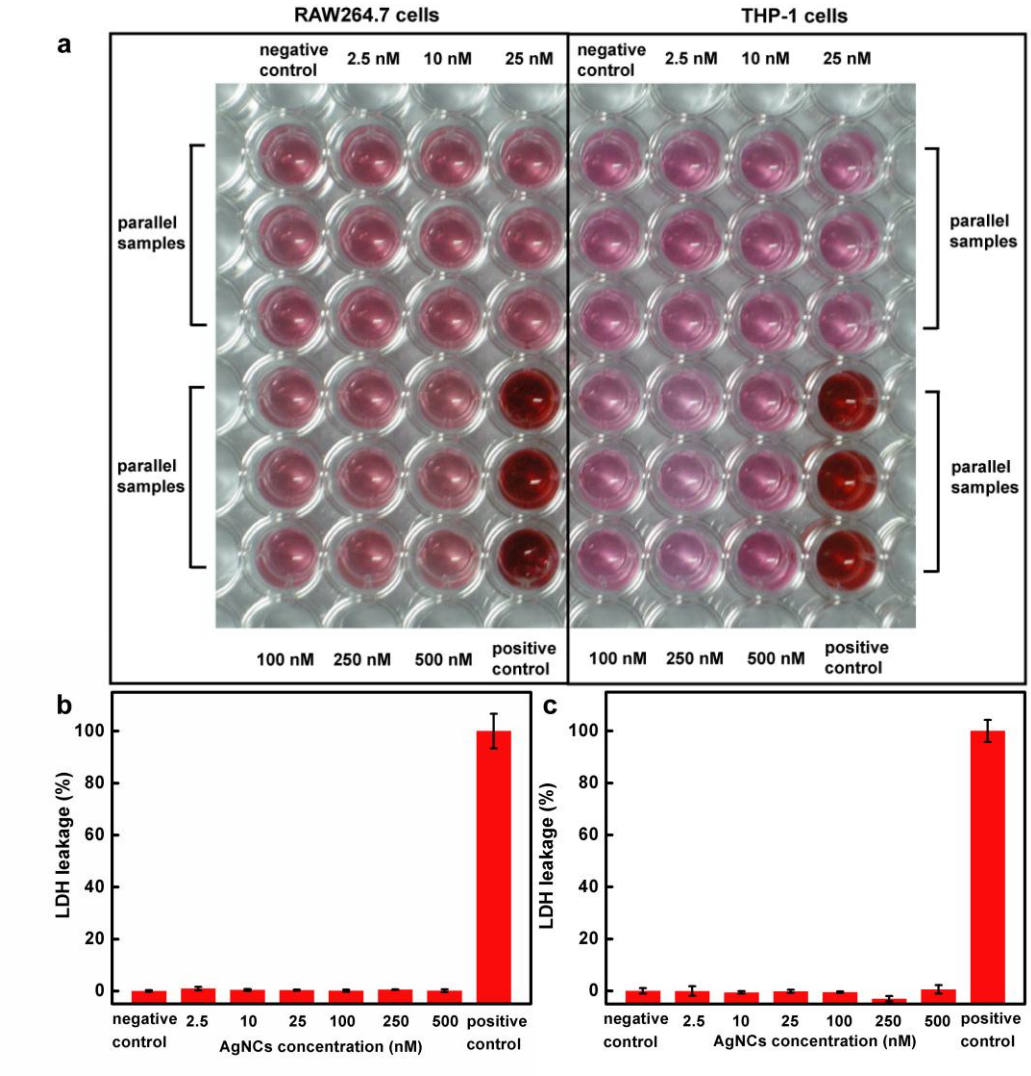

Fig. S6 (a) Image of the toxicity of CpG-AgNCs to RAW264.7 cells and THP-1 cells. Percentage of LDH leakage of RAW264.7 cells (b) and THP-1 cells (c) after incubation with CpG-AgNCs with different concentrations for 48 h. The error bars represent variations among three independent measurements.

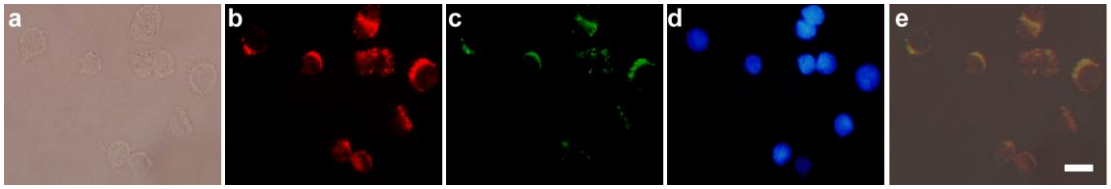

Fig. S7 Confocal images of the FITC-labeled CpG-AgNCs. (a) Bright field image of RAW264.7 cells. (b) CpG-AgNCs exhibit red luminescence. (c) Fluorescence image of FITC-labeled CpG in RAW264.7 cells. (d) The cell nucleus was indicated using Hoechst 33258. (e) Fluorescence image overlay of the four images. Scale bar = 10 μm.

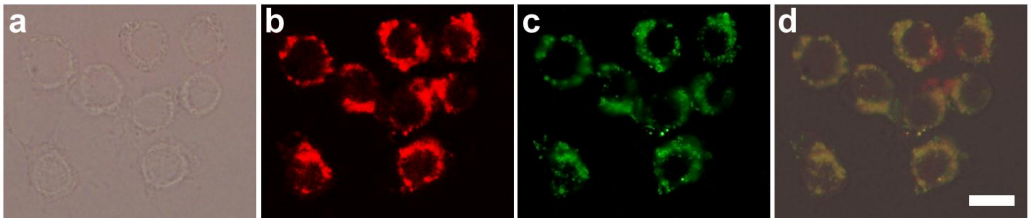

Fig. S8 Co-localization of CpG-AgNCs with Lysotracker green revealed significant sequestration of CpG-AgNCs in endosomes and lysosomes. (a) Bright field image of RAW264.7 cells. (b) CpG-AgNCs exhibit red luminescence. (c) The cells were stained with Lysotracker Green. (d) Fluorescence image overlay of the three images. Scale bar = 10 μm.

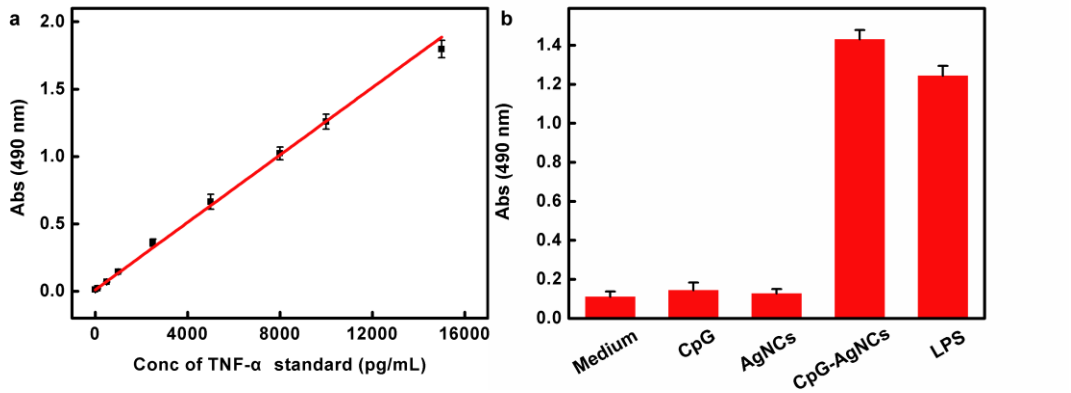

Fig. S9 ELISA results of TNF-α detection. (a) Calibration curve attained by testing of TNF-α standard. (b) Colorimetric absorbance results from ELISA detection of TNF-α in treated samples. The error bars represent variations among three independent measurements.

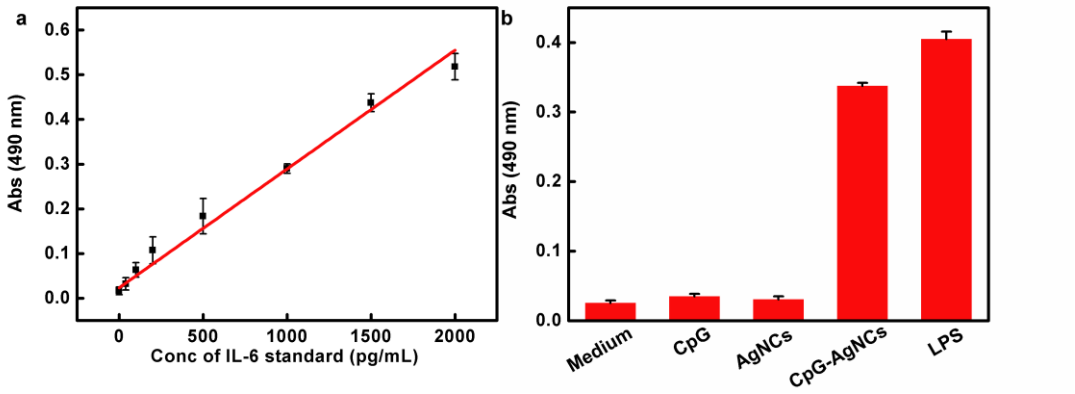

Fig. S10 ELISA results of IL-6 detection. (a) Calibration curve attained by testing of IL-6 standard. (b) Colorimetric absorbance results from ELISA detection of IL-6 in treated samples. The error bars represent variations among three independent measurements.

Table 1. The activities of the released LDH (absorbance at 490 nm) of RAW264.7 cells after incubation with CpG-AgNCs with different concentrations for 48 h.

No.	Absorbance (490 nm)							
	negative control	2.5 nM	10 nM	25 nM	100 nM	250 nM	500 nM	positive control
1	0.365	0.400	0.382	0.377	0.363	0.375	0.379	2.771
2	0.369	0.384	0.370	0.369	0.357	0.377	0.358	2.493
3	0.356	0.367	0.366	0.366	0.377	0.372	0.361	2.746

Table 2. The activities of the released LDH (absorbance at 490 nm) of THP-1 cells after incubation with CpG-AgNCs with different concentrations for 48 h.

No.	Absorbance (490 nm)							
	negative control	2.5 nM	10 nM	25 nM	100 nM	250 nM	500 nM	positive control
1	0.306	0.282	0.284	0.283	0.275	0.208	0.270	2.264
2	0.281	0.319	0.270	0.292	0.280	0.231	0.286	2.122
3	0.268	0.250	0.268	0.268	0.269	0.246	0.330	2.128